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## Apoptosis and autoantibodies in systemic lupus erythematosus

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**NEW INSIGHTS INTO THE PATHOGENESIS OF  
SYSTEMIC LUPUS ERYTHEMATOSUS (SLE):  
the role of apoptosis**

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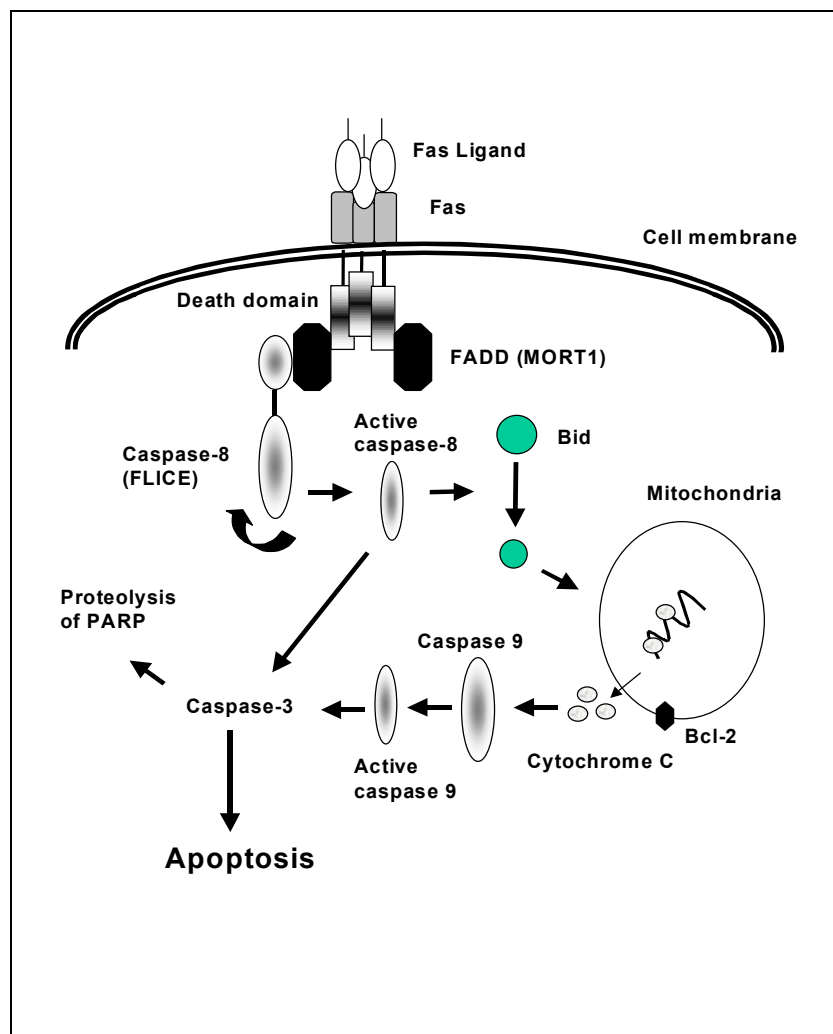
### Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with a wide spectrum of clinical and immunological abnormalities. The prevalence of the disease ranges from 14.6 till 50.8 per 100.000 persons [1]. SLE develops predominantly in women of childbearing age. The presence of autoantibodies, especially those directed to dsDNA, is characteristic for the disease. The cause of SLE is unknown. Insight into the pathogenesis of the disease, however, has deepened in recent years. In particular studies on apoptosis and clearance of apoptotic cells in lupus have shed a new and intriguing light on the development and course of the disease. In this review we will focus on apoptosis, on the elimination of apoptotic cells and on the consequences that disturbances in one of these processes might have on the presentation of autoantigens to the immune system in such a way that tolerance can be broken and autoimmunity occurs.

### Apoptosis

Apoptosis or programmed cell death is recognised as being fundamental to maturation and homeostasis of the immune system. During maturation of the immune system apoptosis of autoreactive lymphocytes in the central lymphoid organs underlies the development of tolerance. Furthermore, due to apoptosis the size of the peripheral lymphoid and myeloid compartments is limited. Activated lymphocytes are deleted through apoptosis following an immune response [2]. Apoptosis is initiated through the ligation of specific death receptors on the cell surface. Ligation of the death receptors is followed by a cascade of enzymatic activations, morphologically accompanied by condensation and fragmentation of cells and nuclei, and blebbing of the plasma membrane [3]. In these blebs autoantigens are exposed. Casciola-Rosen et al. showed that in apoptotic keratinocytes many nuclear and cytoplasmic antigens, that are characteristic targets for autoantibodies, are clustered in high concentrations in the cell membrane [4]. Furthermore, alterations of cellular constituents during apoptotic degradation, occurring via posttranslational modifications such as oxidation, phosphorylation, or citrullination, could induce immunogenicity [5,6]. These findings made it understandable why in autoimmunity an immune response can develop against intracellular, cryptic epitopes, and stresses the important role apoptotic cells might play in the pathogenesis of autoimmune disorders. Whenever apoptotic cells do accumulate by an increased rate of apoptosis, decreased elimination, or a combination of both, it can be imagined that, in the proper environment, tolerance can be broken. For the initiation of the apoptotic process ligation of the cell death receptors is mandatory. Cell death receptors belong to the tumor necrosis factor (TNF) receptor superfamily and share a similar, cysteine rich extracellular domain. In addition, the death receptors contain a homologous cytoplasmic sequence called the death domain. The best characterised death receptors are Fas and TNFR1. The Fas-mediated apoptosis pathway has been extensively

studied and is crucial for the development of immune tolerance and privilege (reviewed in [7] and [8]). Fas, also known as APO-1 or CD95, is a cell surface protein of 45-48 kD [9]. Fas is constitutively expressed on subpopulations of peripheral blood lymphocytes,



**Figure 1:** Apoptotic signal transduction induced by binding of Fas ligand (FasL) to its receptor Fas (CD95). FasL is a homotrimeric molecule. Binding to Fas results in trimerization of Fas. The Fas cytoplasmatic region carries a death domain. Trimerization of this death domain recruits caspase 8 via an adaptor called FADD (Fas associated death domain)/MORT1. Upon recruitment by FADD, caspase-8 (also called FLICE) drives its activation through self-cleavage. Activated caspase-8 then degrades poly (ADP-ribose) polymerase (PARP), an enzyme that is thought to be involved in DNA repair, and activates downstream effector caspases committing the cell to apoptosis with the characteristic degradation of chromosomal DNA and morphological changes. Furthermore, caspase-8 cleaves Bid, a pro-apoptotic member of the BH3 subfamily. Cleaved Bid stimulates the release of cytochrome C from mitochondria which activates caspase-9, which subsequently activates caspase-3. The release of cytochrome C into the cytoplasm can be inhibited by bcl-2 which resides on the cytoplasmic face of the mitochondrial outer membrane.

predominantly memory T cells, and is strongly upregulated on T- as well as B-lymphocytes upon activation [10]. Cross-linking of Fas by Fas ligand (FasL), a membrane glycoprotein of 40 kD, induces trimerization of the receptor. This recruits caspase-8 via an adaptor called FADD/MORT1. Aggregation of caspase-8 causes self-activation and activates pro-caspase-3 by proteolytic cleavage that cleaves various other cellular substrates, which finally results in DNA fragmentation (figure 1). Whether apoptosis is induced after engagement of the Fas receptor depends on many different factors such as the stage of the cell in ontogeny and the state of activation of the cell involved. Many of these factors influence the expression of the Bcl-2 protein. This protein belongs to a family of related cytoplasmic proteins that are key regulators of apoptosis. The family consists of at least 15 members, divided into three subfamilies [11]. The Bcl-2 cohort promotes survival, whereas the Bax and BH3 subfamilies facilitate apoptosis.

Disturbances in one of the many factors that regulate the apoptotic process, might change the balance present in the immune system and may predispose for the development of autoimmune phenomena. In recent years many studies have been performed, both in animal models and in patients with autoimmune diseases, in which the role of these different factors has been investigated.

### **Animal models**

The importance of functionally intact apoptotic pathways for maintenance of immune homeostasis and prevention of autoimmunity was highlighted by some intriguing studies in lupus prone mice. Historically, experimental studies in animals designed to obtain further insight in the pathogenesis of SLE are based on the MRL-lpr/lpr (lymphoproliferation), CBA-lpr<sup>cg</sup>/lpr<sup>cg</sup> and C3H/HeJ-gld/gld (generalised lymphoproliferative disease) mice models. These animals spontaneously develop lymphadenopathy as well as autoimmune features characterised by the presence of autoantibodies to nuclear antigens [12]. It has been recognised that the molecular basis of these abnormalities is a defect in Fas mediated apoptosis. The MRL-lpr/lpr mice have a mutation in the Fas molecule [13] that, in the proper genetic background, results in the generation of abnormally spliced mRNA and a severe reduction in functional Fas mRNA. The C3H/HeJ-gld/gld mice are deficient in functional Fas ligand (FasL) due to a single base mutation [14]. The defects in Fas and FasL, respectively, result in an incomplete elimination of peripheral autoreactive cells as this elimination occurs predominantly by Fas mediated apoptosis [15]. Other evidence for the importance of Fas induced apoptosis in the development of autoimmune disease was delivered by the discovery of soluble Fas [16]. Soluble Fas (sFas) is the transcript of Fas mRNA lacking the transmembrane domain because of the deletion of the exon encoding this region. Injecting sFas into female mice of a certain mouse strain (CD1) resulted in the development of autoimmune features due to blocking of Fas induced apoptosis [16]. The importance of Bcl-2 expression for lymphocyte survival and the development of autoimmune disease was supported by

experiments with transgenic mice. In a model of host-versus-graft disease, a self-limited model of systemic autoimmune disease, it was shown that overexpression of Bcl-2 provides survival signals for autoreactive B cells. This resulted in the production of pathogenic autoantibodies [17]. Furthermore, it has been shown that Bcl-2 protects thymocytes from radiation and glucocorticoid-induced cell death [11].

In summary, data from animal models demonstrate that alterations in apoptosis induction can contribute to the development of autoimmunity. In particular, lack of induction of apoptosis of autoreactive lymphocytes can induce autoimmunity in certain animal models.

### Human SLE

Following the demonstration that the autoimmune lymphoproliferative syndrome in the *lpr* and *gld* mouse strains was based on a single gene defect, studies in humans were performed to detect similar functional defects in Fas or FasL. Indeed, some families have been described with a mutation in Fas [18-21] or FasL [22]. Also in humans, defects in the Fas apoptotic pathway result in splenomegaly and lymphadenopathy called the autoimmune lymphoproliferative syndrome (ALPS), formerly named the Canale-Smith syndrome [2,18]. In contrast to the mouse models, autoimmune phenomena in affected human individuals are rare [20]. Moreover, in two studies a total amount of 218 lupus patients were screened for a defect in the FasL gene. This revealed only one affected patient suggesting that such mutations are an uncommon cause of the disease [22,23]. Additional studies performed to detect functional defects in the apoptotic machinery in SLE did not demonstrate any abnormalities [24,25]. Several studies have been performed in which sFas was measured in SLE patients. These studies demonstrated elevated levels of sFas in lupus patients and a relation between sFas levels and disease activity [26-32]. Elevated levels of sFas were found prior to relapse of the disease [32] and correlated with the state of activation of B-lymphocytes [31]. In contrast to the study of Cheng et al. in which it was shown that sFas inhibited Fas-mediated apoptosis, the presence of sFas does not seem to block Fas-induced apoptosis in significant amounts in SLE patients. Levels of apoptotic lymphocytes in SLE patients in vitro as well as in vivo were higher than in healthy controls [24,33-35]. Even in patients with elevated levels of sFas increased numbers of apoptotic lymphocytes could be demonstrated in the peripheral blood [36]. Probably, levels of sFas reflect increased lymphocyte activation. It has been shown that even during inactive disease increased proportions of activated T- as well as B-lymphocytes are present in the peripheral blood of SLE patients [31,37]. Probably, this is most pronounced in patients with grumbling disease at risk for a relapse. During disease exacerbations the state of lymphocyte activation increases further [37]. Lymphocyte activation results in increased transcription of membrane Fas as well as the alternative splice form sFas, and explains the relation found between sFas levels and disease activity. This suggests that, in the long term, an association between sFas levels and organ damage will occur in patients with remitting disease activity. Indeed, sFas levels correlated with

organ damage as measured by the SLICC/ACR score [38]. The hypothesis that sFas is just a reflection of lymphocyte activation and is therefore not specific for SLE is supported by the presence of elevated sFas levels in rheumatoid arthritis patients [28,36]. Finally, it must be stressed that the relation between lymphocyte activation, sFas and apoptosis is complex, especially in SLE patients. Many factors influence this interrelation. Increased expression of the aforementioned proto-oncogenes such as Bcl-2 have been found in SLE [39-41]. Furthermore, increased levels of soluble Fas ligand [42], and the presence of antibodies to poly (ADP-ribose) polymerase (PARP) can influence the results in studies dealing with apoptosis in SLE. PARP is necessary for the repair of DNA breaks and is inactivated early in apoptosis in order to enable the execution of the apoptotic machinery. This inactivation is blocked by the presence of antibodies to PARP, which have been detected in SLE patients [43].

So, although suggested by animal studies, no genetically defined defects in the major, Fas-mediated, apoptotic pathway have been detected in human SLE. Therefore, a hereditary defect in the induction of apoptosis as a mechanism of elimination of autoreactive lymphocytes does not seem a prerequisite factor for the development of human autoimmune disorders. In contrast, studies in human SLE have demonstrated increased levels of apoptotic cells even in the presence of factors that can inhibit apoptosis induction like elevated levels of sFas and increased expression of Bcl-2. The increased levels of apoptotic cells found might be due to increased in vivo activation and Fas-mediated apoptosis of lymphocytes. Recently, it was found that chlorpromazine, a drug known to induce lupus erythematosus, induces apoptosis in lymphoblasts independently of Fas [44]. Taken together, data from lupus patients supply evidence that the increased induction of apoptosis via Fas or via other pathways can trigger the development of autoimmunity.

### **Phagocytosis of apoptotic cells**

The increased presence of apoptotic cells as demonstrated in the peripheral blood of SLE patients can be accounted for by an increased level of activation induced cell death. However, apoptosis is a physiological mechanism and occurs continuously in impressive amounts. For example, every day  $2 \times 10^9$ /kg body weight apoptotic neutrophils are removed from the blood stream [45]. Removal of apoptotic cells occurs very effectively via phagocytosis by bystander (semi-professional) or professional phagocytes like monocytes and macrophages [27]. Rapid elimination of apoptotic cells is important as it prevents the release of (toxic) cell constituents like cytolytic enzymes. Furthermore, it has become clear that during the process of apoptosis antigens are newly exposed in the cell membrane of the apoptotic cell [4]. Adequate removal of apoptotic cells therefore also seems important for the prevention of (excessive) autoantigenic exposure.

The interaction between apoptotic cells and other cells, necessary for their elimination, is very complex [46]. Monocytes and macrophages constitutively express several receptors

like CD14, CD36 and scavenger receptors, all involved in the recognition, binding and internalisation of apoptotic cells [46-55]. Next to binding of apoptotic cells to these receptors several serum proteins play a role in this process of elimination (figure 2). Already early in the apoptotic cascade the cell membrane changes. For example, phosphatidylserine is exposed at the outer surface of the cell membrane. Due to these changes several serum constituents like complement C1q, C3 and C4, C-reactive protein (CRP), serum amyloid protein P (SAP) and phospholipase A2 can bind to the apoptotic cell and facilitate, by yet unknown mechanisms, the interaction with phagocytes [56-59]. Furthermore, phagocytosis itself modulates phagocyte behaviour. The ingestion of apoptotic cells in vitro promotes the secretion of anti-inflammatory cytokines in human macrophages [60]. Furthermore, uptake of apoptotic neutrophils by macrophages reduced the uptake of the former cells when a rechallenge was performed after 48 hours [61]. In this context, it can be speculated that an increased rate of apoptosis could lead to an overflow of the phagocytic system with apoptotic cells through this negative feedback loop.

Thus, as will be discussed, next to increased induction of apoptotic cells an intrinsic decreased clearance capacity of the phagocytic system, possibly in combination with defects in the production of anti-inflammatory mediators by macrophages, might be an important pathogenic factor in the development of SLE.

### **Animal models**

Several animal studies have deepened our understanding of the role of apoptotic cells and disturbances in the clearance of these cells for the development of autoimmune phenomena. First, it has been shown that the presence of large numbers of apoptotic cells can evoke an immune response. Mevorach et al. demonstrated that the intravenous injection of apoptotic thymocytes resulted in the production of autoantibodies to nuclear antigens in the majority of normal mice [62]. In addition, the consequences of a disturbance in the removal of apoptotic cells have been addressed in several studies. As discussed before, several serum proteins bind to the surface blebs of apoptotic cells. C1q binds to apoptotic keratinocytes [59]. SAP can bind to extracellular dsDNA and chromatin, also present in the apoptotic blebs, under physiological circumstances [56]. The functional impact of these findings has subsequently been demonstrated in mice using selective gene deletion [63-66]. The important role C1q plays in the removal of apoptotic cells was demonstrated in C1q-knock out mice. C1q<sup>-/-</sup> mice had higher titres of autoantibodies and higher mortality compared with strain-matched controls. Furthermore, 25% of C1q<sup>-/-</sup> mice had glomerulonephritis with immune deposits and multiple apoptotic cell bodies. In C1q<sup>-/-</sup> mice without glomerulonephritis, significantly greater numbers of glomerular apoptotic bodies were detected than in controls [64]. Similar findings have been reported in SAP-deficient mice. In mice with a targeted deletion of the SAP gene autoimmune disease characterised by the presence of autoantibodies to DNA and



chromatin and severe glomerulonephritis developed spontaneously [65]. Further indications that the removal of apoptotic cells and their antigenic structures is relevant in the pathogenesis of SLE are delivered by a recent study performed with Dnase1-deficient mice [67]. Dnase1 might have a protective task in the removal of DNA from nucleoprotein complexes so preventing immune stimulation. Indeed, deletion of Dnase1 resulted in the occurrence of classical symptoms of SLE, including the production of anti-nuclear antibodies and the development of glomerulonephritis.

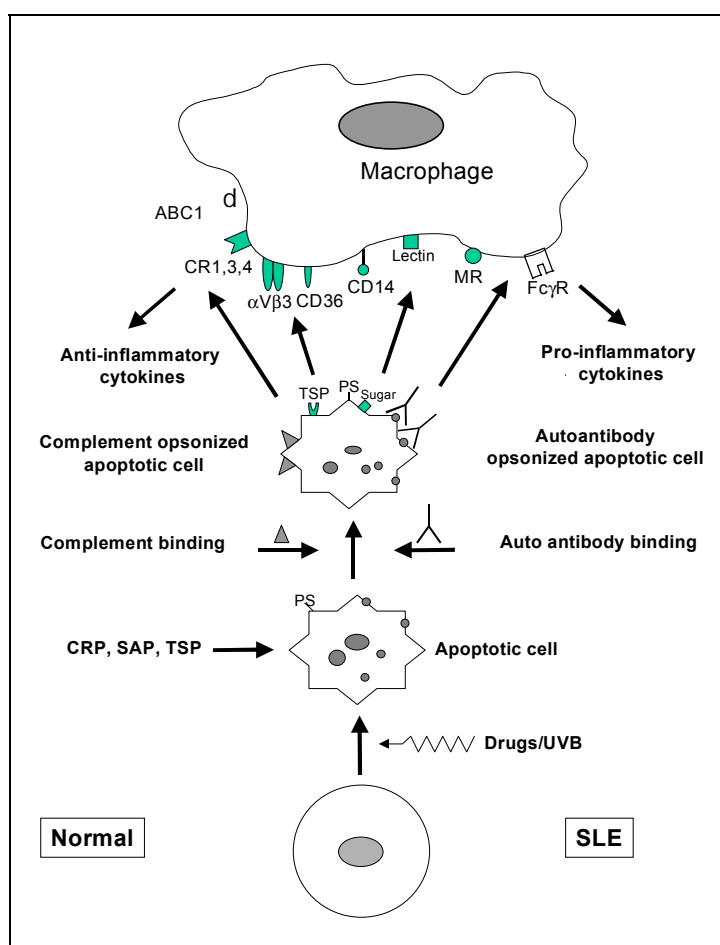
### Human SLE

Evidence that disturbed phagocytosis of apoptotic cells might be a relevant factor in the pathogenesis of human autoimmune disease was demonstrated by Hermann et al [68]. They showed that phagocytosis of apoptotic cells by in vitro differentiated macrophages obtained from SLE patients was impaired compared to phagocytosis by macrophages obtained from healthy controls.

Interestingly, in families with congenital deficiencies of the complement factors C1q, C2 or C4, the vast majority of the affected members spontaneously develop SLE [69]. The important role of complement factors is further supported by data showing that the addition of complement components to an in vitro phagocytosis assay using human monocyte-derived macrophages and apoptotic lymphocytes provided a more than threefold increase in the uptake of apoptotic cells [70]. Next to deficiencies in the complement factors also the CRP response in SLE patients seems to be impaired [71]. Recently, we studied whether in human SLE SAP production is (relatively) deficient compared to disease controls and healthy controls but could not find significant differences [72]. Further studies are underway to investigate whether SAP is functionally intact and influences phagocytotic capacity in SLE patients. Finally, Napirei et al. measured Dnase1 activity in the sera of SLE patients and found lower levels than in normal controls [67].

As pointed out, next to a decrease in the concentrations of serum proteins with a functional role in the process of phagocytosis of apoptotic cells, one might hypothesise that changes in the expression of membrane receptors necessary for binding and internalisation of apoptotic cells can influence the phagocytotic capacity. Indeed, monocytes in SLE patients expressed significantly lower levels of CD14 [73].

Interestingly, this receptor mediates clearance of apoptotic cells without inciting inflammation [54,74]. A lowered expression of CD14, therefore, can tilt phagocytosis of apoptotic cells, which normally is non-inflammatory, towards an inflammatory process in SLE patients. In addition, apoptotic cells can be opsonised by autoantibodies present in patients with autoimmune disease which are directed to auto-antigens presented on apoptotic cells [75,76]. Ligation of the Fc fragment of these antibodies with Fc $\gamma$ -receptors on macrophages then induces the secretion of pro-inflammatory cytokines, so contributing to the process of inflammation which is characteristic for autoimmune diseases (figure 2).



**Figure 2:** Phagocytosis of apoptotic cells by macrophages. Cells die by apoptosis during tissue turnover or at the end of an immune response. In SLE patients additive stimuli like UVB (inducing apoptotic keratinocytes) or certain drugs (like chlorpromazine, inducing apoptosis in lymphoblasts) can increase the number of apoptotic cells generated. Cells undergoing apoptosis display an orderly process of nuclear condensation and fragmentation and cytoplasmic contraction. Phosphatidylserine (PS) that normally resides at the inside of the cell membrane, flips to the outside to become exposed on the cell membrane. Furthermore, surface blebbing and packaging of cellular components within membranes occurs before their budding from the cell. In the smaller surface blebs fragmented endoplasmic reticulum and ribosomes are concentrated. The larger blebs contain nucleosomal DNA. In SLE patients autoantibodies can bind to these antigens whenever exposed. This will enable Fc $\gamma$ R-bearing cells to interact with antibody opsonized apoptotic cells via the Fc $\gamma$ R resulting in the release of pro-inflammatory cytokines. Without the presence of autoantibodies the apoptotic cell is only opsonized with SAP, complement proteins and other serum factors like thrombospondin (TSP). Interaction of these molecules facilitates non-inflammatory phagocytosis mediated by a variety of membrane receptors on phagocytes like the ATP binding cassette transporter ABC1, complement receptors (CR1, 3, 4), the vitronectin receptor ( $\alpha v\beta 3$ ), CD36, CD14, lectins, and the mannose receptor (MR).

In their study on binding of anti-phospholipid antibodies to apoptotic cells, Manfredi et al. found massive secretion of TNF alpha when apoptotic cells opsonised with these antibodies were internalised by macrophages [75].

### Conclusion

There is increasing evidence that the presence and accumulation of apoptotic cells can result in autoimmunity. Whether this accumulation in SLE patients is due to increased production of apoptotic cells, results from decreased phagocytic capacity, or from the combination of both has to be proven. Nevertheless, it has been shown that tolerance can be broken due to increased amounts of apoptotic cells. Alternatively, or in conjunction, posttranslational modifications occurring during the process of apoptosis of cellular antigens can bypass tolerance. Breaking tolerance induces the production of autoantibodies that will bind to their antigens whenever exposed on the cell membrane. The presence of these autoantibodies will allow interaction with Fc gamma receptor binding cells. This will then result in Fc receptor mediated phagocytosis, which induces the release of pro-inflammatory cytokines. Finally, this cascade of events will lead to the development of inflammation characteristic for many autoimmune diseases. Understanding the processes underlying these inflammatory lesions will allow us in the near future to intervene therapeutically much more specific in autoimmune mediated disorders so reducing morbidity and mortality of patients suffering from these diseases.

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